seemed to be so strong that they never regained the ability to suck even after they were transferred to untreated plants. Although they produced a few offspring before death, the offspring were also unable to feed and died without growing. These actions of R-768 are different from the conventional insecticides, suggesting that R-768 has a novel mode of action. In the laboratory study, R-768 was equally effective against organophosphate-, carbamate- and pyrethroid-resistant strains of aphid as well as against susceptible strains of *M persicae* and *A gossypii* (Table 2). This biological performance is also consistent with a new mode of action.

In a field trial on potatoes, the efficacy of R-768 at 50 or 100 g ha^{-1} against the strain of *A gossypii* that was resistant to lambda-cyhalothrin and pirimicarb, was comparable to that of imidacloprid at 50 g ha^{-1} (Fig 5). These results indicated that R-768 possesses no cross-resistance to other insecticides and it will be useful as a candidate in areas where resistance problems become more serious. R-768 aso showed excellent control of aphids on wheat (Fig 6), on apple trees (Fig 7) and on various crops, without phytotoxicity, in the field trials.

On the other hand, R-768 was safe to beneficial insects such as *Bombyx mori* L, *Osmia cornifrons*, *Bombus terrestris* and *Apis mellifera* L, and nontarget arthropods such as *Harmonia axyridis*, *Chrysopa nipponensis*, *Orius* sp, *Aphelinus* sp, *Ephedrus japonicus* and *Misumenops tricuspidatus* in laboratory studies. This outstanding selectivity was also demonstrated for the pollinators of tomato and strawberry in a greenhouse study, and for some natural enemies in the field trials. Therefore R-768 could be one of a few aphid control agents which can be useful soon after releasing the pollinators and natural enemies in biological control systems.

In conclusion, R-768 is a representative of a new and highly active class of aphid control agent with large margins of safety to beneficial insects and natural enemies. Lack of cross-resistance indicates that R-768 could be useful as a candidate in the resistant management strategies. Its favorable characters make it especially suitable for IPM programmes.

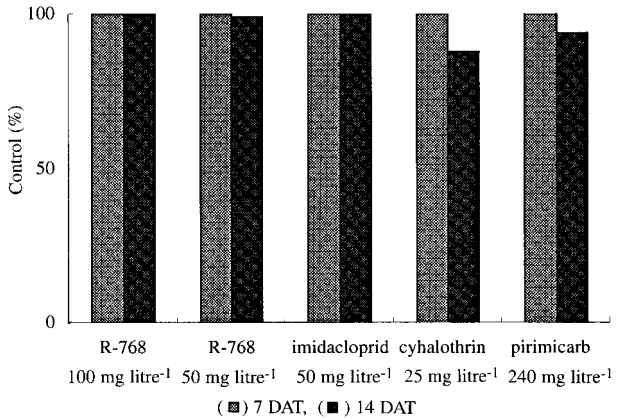

Figure 7. Efficacy of R-768 against *Aphis citricola* on apple trees in a field trial. Application: 22 May 1998 in Japan. Spray: 1500 litre ha^{-1}.

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Expression of *Neurospora crassa* β-tubulin, target protein of benzimidazole fungicides, in *Escherichia coli*

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Abstract: β-Tubulin of a wild-type *Neurospora crassa* strain was expressed using pET-16b, pET-29a(+), and pET-32a(+) expression vectors in *Escherichia coli* BL21 (DE3) strain. Yield of the expressed soluble protein was estimated to be about 0.1 mg ml^{-1} culture broth. The β-tubulins with S-Tag expressed by pET-29a(+) and pET-32a(+) bound to the S-protein Agarose by affinity binding but the thrombin and enterokinase treatments did not release β-tubulin, suggesting that the protease cleavage sites connecting S-Tag and β-tubulin were not exposed to approach of the proteases. The β-tubulin expressed by pET-16b did not bind to nickel resin, suggesting that its His-Tag was folded into the protein core. The protein expressed by pET-32a(+) was bound to the nickel resin and purified by the column chromatography.

Keywords: β-tubulin; gene expression; *Neurospora crassa: Escherichia coli;* expression vector

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1 INTRODUCTION

β-Tubulin is a target protein of benzimidazole fungicides and *N*-phenylcarbamates, such as diethofencarb.[1,2] The three-dimensional structure of the β-tubulin–fungicide complex has not been reported, although amino acids which are involved in the complex formation were elucidated by studies of β-tubulin genes of mutant fungal strains with reduced sensitivity to the fungicides.[3] We have constructed expression systems of a fungal β-tubulin aiming at a nuclear magnetic resonance (NMR) analysis of the β-tubulin–fungicide complex, which will require a milligram-level quantity of the protein with ^{15}N- and/or ^{13}C-labelled amino acids.

2 EXPERIMENTAL METHODS

β-Tubulin cDNA of a wild-type *Neurospora crassa* Spear & Dodge strain was cloned into pET-16b, pET-29a(+), and pET-32a(+) expression vectors after making restriction enzyme sites for cloning at 5′- and 3′-terminals of the coding region of the gene by polymerase chain reaction (PCR). *Nde*I/*Xho*I site was used for cloning into pET-16b, and *Eco*RI/*Sal*I site for cloning into pET − 29a(+) and pET-32a(+). *Escherichia coli* Castell & Chalm BL21 (DE3) strain was transformed by the vector with the β-tubulin gene. The transformant was inoculated in LB medium (1.5 ml) with 50 μg ml^{-1} ampicillin for pET-16b and pET-32a(+) or with 30 μg ml^{-1} kanamycin for pET-29a(+) and incubated at 37°C for 17 h on a shaker at 200 rev min^{-1}. The *E coli* cells were collected by centrifugation and suspended in fresh LB medium (1 ml), 0.1 ml of which was inoculated in LB medium (100 ml) with 500 μg ml^{-1} ampicillin for pET-16b and pET-32a(+) or with 30 μg ml^{-1} kanamycin for pET-29a(+). Preincubation was carried out at 37°C for 2 h on a rotary shaker at 170 rev min^{-1}. The cells were then collected by centrifugation and suspended in the above medium (100 ml) with 1 mM isopropyl-β-D(−)-thiogalactopyranoside (IPTG) to induce the gene expression. They were incubated at 15°C for 20 h at 170 rev min^{-1}. The cells were collected by centrifugation and destroyed by ultrasonic treatment (Ohtake sonicator 5202; 5 × 10 s) on ice. The cell lysate was divided into soluble and insoluble fractions by centrifugation at 18,000 *g* for 10 min, and proteins in the fractions were separated by SDS-polyacrylamide gel electrophoresis on a 5–20% gradient gel (10 × 10 cm) at 20 mV for 2 h. Protein bands on the gel were transferred to a PVDF membrane at 60 V for 4 h for Western blotting and incubated with mouse monoclonal anti-β-tubulin (Amersham) as a primary antibody and then with peroxidase-conjugated goat anti-mouse immunoglobins (Cappel) as a secondary antibody. β-Tubulin on the membrane was detected by treatment with 4-chloro-1-naphthol in the presence of hydrogen peroxide.

The soluble fraction of the lysate containing 0.1 mg of β-tubulin with S-Tag expressed by pET-29a(+) or pET-32a(+) was dissolved in Tris-HCl buffer (20 mM) pH 7.5), containing NaCl (150 mM) and Triton X-100 (1 g litre^{-1}; 1 ml), and incubated with S-protein Agarose 50% slurry (0.2 ml) at 20°C for 3 h. The S-protein Agarose bound with the β-tubulin expressed from pET-29a(+) was then incubated with biotinylated thrombin (5 units; Novagen) in Tris-HCl buffer 20 mM (pH 8.4), containing NaCl (150 mM) and $CaCl_2$ (2.5 mM) at 20°C for 6 h to release β-tubulin. The S-protein Agarose bound with the β-tubulin from pET-32a(+) was incubated with recombinant entrokinase (1 unit; Novagen) in Tris-HCl buffer (20 mM; pH 7.5) containing NaCl (150 mM) and Triton X-100 (1 g litre^{-1}) at 20°C for 17 h.

The soluble fraction of the lysate containing 20 μg of β-tubulin with His-Tag expressed by pET-16b or pET-32a(+) was dissolved in Tris-HCl buffer (20 mM; pH 7.9) containing NaCl (0.5 M) and imidazole (5 mM; 1 ml) and passed through a nickel resin column (0.5 ml) equilibrated with the same buffer for purification of the expressed protein. Proteins bound to the column were eluted with Tris-HCl buffer (20 mM; pH 7.9) containing NaCl (0.5 M) and imidazole (1 M; 3 ml) after washing the column with Tris-HCl buffer (20 mM; pH 7.9), containing NaCl (0.5 M) and imadazole (60 mM; 3 ml).

3 RESULTS AND DISCUSSION

A pronounced band of a protein of 60 kDa, which corresponds to the molecular mass of β-tubulin expressed with a tag for purification, was detected not only in the insoluble fraction but also in the soluble fraction of the cells containing the β-tubulin gene in pET-16b or pET-29a(+) after the overnight incubation with 1 mM IPTG at 15°C (Fig 1-A, B). This band was, however, faint in those of control IPTG-untreated cells (Fig 1-A, B). The band of a protein expressed from pET-32a(+) was at 70 kDa (Fig 1-C), which corresponds to the molecular mass of β-tubulin with S-Tag, His-Tag, and thioredoxin as a fused protein. Incubation with 1 mM IPTG at 20°C for 17 h or at 25°C for 4 h resulted in expression of only insoluble protein as inclusion bodies. The bands of expressed proteins in the fractions of IPTG-treated cells were confirmed to contain β-tubulin by coloration in the Western blotting. Yield of the expressed soluble proteins was estimated to be about 0.1 mg ml^{-1} culture broth.

The β-tubulins with S-Tag expressed by pET-29a(+) and pET-32a(+) bound to the S-protein Agarose by affinity binding, but the thrombin and enterokinase treatments did not release β-tubulin, suggesting that the cleavage sites connecting S-Tag and β-tubulin were not exposed to approach of the proteases.

The β-tubulin with His-Tag expressed by pET-16b did not bind to the nickel resin, suggesting that the His-Tag was folded into the protein core. The protein expressed from pET-32a(+) was bound

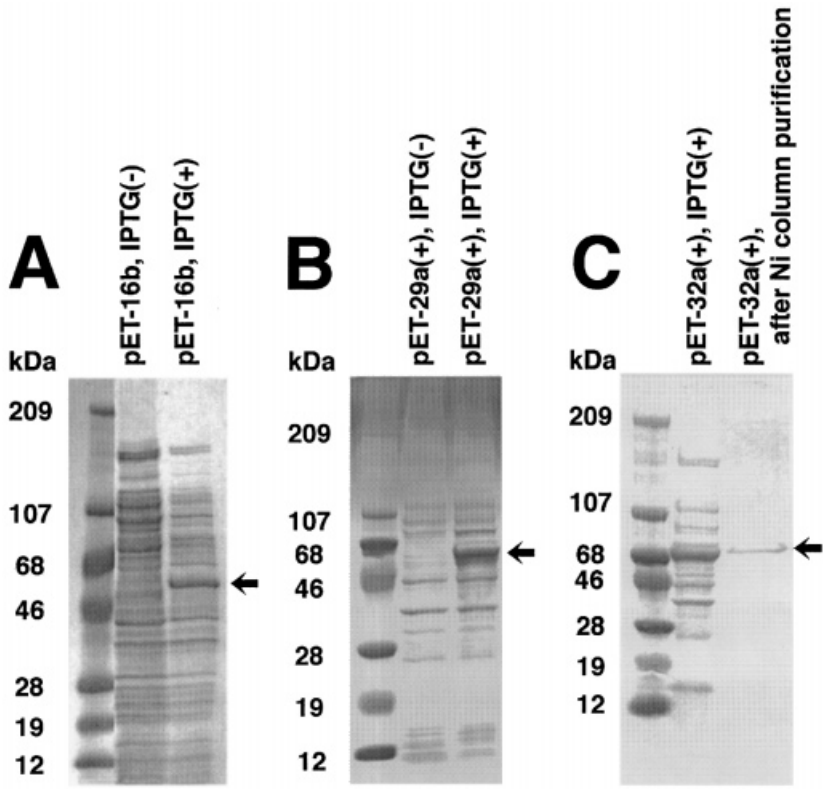

Figure 1. SDS-polyacrylamide gel electrophoresis of soluble fractions of *Escherichia coli* lysate expressing *Neurospora crassa* β-tubulin (A) by pET-16b; (B) by pET-29a(+); (C) by pET-32a(+). IPTG(−) means lysate of *E. coli* incubated without ITPG, and IPTG(+) lysate of *E. coli* incubated with IPTG. Arrows indicate bands of expressed proteins.

to the nickel resin and eluted with the buffer containing 1 M imadazole. The eluted fraction contained only one band of protein on the SDS-polyacrylamide gel electrophoresis (Fig 1-C). The yield of the expressed protein after this purification was about 10% of the total protein expressed in soluble fraction.

4 CONCLUSION

We succeeded in expressing a fungal β-tubulin, which is contained in a very small amount in fungal cells and difficult to purify, in a large amount using the pET expression vector systems in *E coli*. The protein expressed using pET-32a(+) was purified by nickel resin column chromatography. This will open the way to obtaining β-tubulin for three-dimensional structure analysis of the complex with fungicide by NMR. Codon 198 of the β-tubulin cCDN has been altered from GAG (Glu) to GGG (Gly) by a site-directed mutagenesis using PCR (unpublished data). This single base mutagenesis was reported to give a benzimidazole-resistant, diethofencarb-sensitive-type β-tubulin.[4] We have started cloning of the mutated β-tubulin gene into an expression vector for a study to compare the binding mode of benzimidazole fungicides with that of diethofencarb to β-tubulin.

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Gene disruption and biochemical characterisation of 3-isopropylmalate dehydrogenase from *Stagonospora nodorum*

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Abstract: This summary reports work with auxotrophs of the pathogenic fungus *Stagonospora nodorum*, the causal agent of leaf spot and glume blotch in wheat and barley, with a view to investigating the biochemical basis of pathogenicity. Biosynthetic enzymes may serve as targets for novel fungicides and genetic target validation can identify new candidates for a biochemical approach to fungicide discovery.

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